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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: AUTOINDUCER

$$R-NH-\underset{\underset{X}{\parallel}}{\underset{|}{C}}\overset{(CH_2)_n}{\diagup}Y$$

(I)

(57) Abstract

The compound N-(β-ketocaproyl)L-homoserine lactone is shown to be an autoinducer that enhances gene expression in a wide variety of microorganisms. Use can be made of this property for diagnostic purposes, e.g. when gene expression causes bioluminescence or antibiotic production, or to promote bacterial growth. The invention claims use for these purposes of the compound and analogues of formula (1) where n is 2 or 3, each of X and Y is O, S or NH, and R is optionally-substituted C1-C12 alkyl or acyl. Some of these are also claimed as new compounds.

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AUTOINDUCER

5   An autoinducer is a chemical molecule, often
quite a small one, which is produced by a microorganism
during metabolism and which then acts to increase the
expression of genes of the microorganism.

          N-(β-ketocaproyl) homoserine lactone [3-oxo-
10   N-(tetrahydro-2-oxo-3-furanyl) hexanamide (formula 2)
has long been recognised as an autoinducer regulating
expression of lux genes and hence the bioluminescent
phenotype in the marine microorganism, Vibrio fischeri
(Eberhard et al., 1981). Although characterised as a
15   bacterial pheromone (Eberhard, 1972), it has been
identified only in the one species of bioluminescent
bacteria and any broader role in signalling, such as
communicating nutritional viability to other bacteria
(Eberhard et al., 1981) has previously had no
20   foundation in scientific evidence. It has been
observed that the autoinducer of Vibrio fischeri is
similar in structure to A-factor (formula 4), a
regulatory molecule which is produced by Streptomyces
griseus (Silverman et al., 1989; Meighen, 1991) and
25   which causes a self-induction of sporulation and
streptomycin synthesis. Intellectually, this has been
assimilated by several workers as suggestive of a
broader role for such molecules. To establish
precisely the current level of understanding, we quote
30 . from two recent reviewers:

          "Perhaps this chemical relationship is an
indication that mechanisms used by bacteria to sense
their environments have a common origin and that there
is a large class of signalling molecules or 'bacterial
35   hormones' similar in structure and mode of action."
(Silverman et al., 1989).

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"The possibility that the lux autoinducers are part of a larger class of signalling molecules (allomones, pheromones or hormones) used to sense the local nutritional or chemical environment has been

5   suggested." (Meighen, 1991).

While these two statements establish the possibility of a large class of signalling molecules, it must be re-emphasised that there is no basis in experimentation for confirming the above hypothesis and

10   certainly no indication that N-(β-ketocaproyl) homoserine lactone (formula 2) may be directly involved in gene regulation of microorganisms other than Vibrio fischeri and the closely related Vibrio logei. In addition, a study of other bacteria, including

15   nonluminous species, for their ability to provide autoinducer for the related Vibrio harveyi lux system (N-β-hydroxybutyryl homoserine lactone) (formula 3) failed to identify any terrestrial sources for complementation (Greenberg et al., 1979).

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Formula 2

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Formula 3

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35   Formula 4

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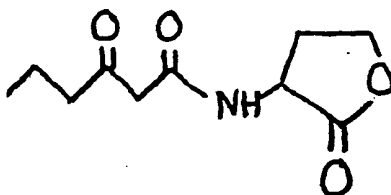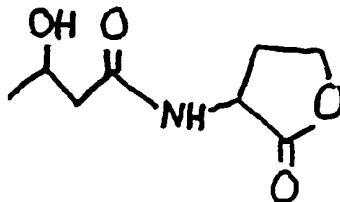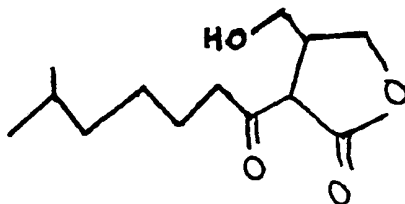
In a research programme directed at the study
of carbapenem synthesis in prokaryotes, we have
recently discovered that the compound of formula 2
regulates the expression of carbapenem synthesis in
5   Erwinia. It appears that there is a family of
compounds, including those of formulae 2 and 3 and
analogues thereof, which control (increase or decrease)
gene expression in a variety of microorganisms. This
family is sometimes hereinafter referred to as "N-(β-
10   ketocaproyl) homoserine lactone or analogue". Some of
this family of compounds, including specifically those
of formulae 2 and 3, are produced by various
microorganisms for which they act as autoinducers.
We have synthesised various members of this
15   family of compounds, including optically active isomers
thereof. This invention includes as new compounds
those members of the family that have not been
previously described. The invention also includes use
of the compounds of the family to control gene
20   expression in microorganisms. Some of the practical
implications of this use are discussed below.
Thus in one aspect the invention provides use
of a compound having the formula 1 to control gene
expression in microorganisms other than V. fischeri, V.
25   logei and V.harveyi, wherein formula 1 is

$$R-NH-\overset{\displaystyle (CH_2)_n}{\underset{\displaystyle \underset{\displaystyle X}{\|}}{\diagdown}}Y$$

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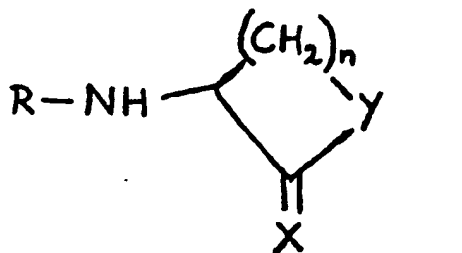
where n is 2 or 3
Y is O, S or NH
X is O, S or NH
35   R is C_1 - C_{12} alkyl or acyl which may be
substituted.

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Preferably n is 2, so that the ring is five-
membered. Although it is not necessary for Y and X to
be the same, both are preferably O as in the compounds
of formulae 2 and 3. Preferably R is C2 to C6 acyl.
5 Preferably R carries a keto or hydroxy group in the β
position.

In another aspect, the invention provides, as
new compounds, optically active isomers of the
compounds of formula 1 as defined above. Preferably
10 the optically active isomers are L-isomers, since these
have proved more active than their corresponding D-
isomers at increasing gene expression. The D-isomers
may be useful to inhibit gene expression.

Use is made of these compounds to control
15 gene expression in microorganisms. The control
exercised may be to decrease, but is more usually to
increase, gene expression. The microorganisms
concerned include bacteria, both Gram negative and Gram
positive, yeasts and fungi. It is at the basis of the
20 invention that a wide variety of microorganisms have
some gene whose expression is affected in some way by
at least one compound within the family. This control
technique often involves the use of microorganisms that
are not themselves capable of producing an autoinducer
25 within the family of compounds, but which are capable,
in the presence of exogenous autoinducer, of expressing
a gene, generally in an easily detectable manner. Two
examples of such microorganisms, which are discussed in
more detail below, are:
30   - A genetic construct pSB237. This has the
capacity to express a bioluminescent phenotype in
E.coli, but only in the presence of added inducer,
preferably the compound of formula 2.
           - A mutant strain B10002/mu22, made by
35 mutation of Erwinia carotovora ATCC3948. Unlik its
parent, the mutant 22 is capable of synthesising

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carbapenem antibiotic only in the presence of added
inducer, such as the compound of formula 2.

In one example of such technique, the
invention provides a method of testing for N-(β-
5   ketocaproyl) homoserine lactone or analogue in a
sample, by incubating the sample in contact with test
bacteria chosen for enhancement of gene expression by
the lactone or analogue, and detecting the gene
expression as a test for the lactone. The limits of
10   detection using a bioluminescent phenotype derived from
E.coli [pSB237] depend upon the time of exposure of the
bacterial culture to inducer. As shown in Example 4
below, at concentrations above 10 ng/ml of culture,
induction of bioluminescence takes less than 10
15   minutes. At lower concentrations induction is
progressively slower, but concentrations of as low as
80 pg/ml can be distinguished from a zero concentration
control after some 20 hours of incubation.

In another example of this technique, the
20   invention provides a method of testing for the presence
in a sample of a first bacterium known to generate N-
(β-ketocaproyl) homoserine lactone or analogue under
particular conditions, which method comprises
incubating the sample under the particular conditions
25   in contact with a test bacterium chosen for enhancement
of gene expression by the lactone or analogue, and
detecting the gene expression as a test for the first
bacterium.

Such a test may be for bacteria generally, or
30   for specific bacteria known to produce autoinducer
under particular conditions. For example, Pseudomonas
aeruginosa produces autoinducer (formula 2) only under
defined growth conditions (see Example 4), specifically
the use of limiting or minimal growth media
35   supplement d with Fe(III). Similarly, there is
preliminary evidence that Listeria monocytogenes

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produces a lactone or analogue only aft r exposure to
low temperature. These characteristics of particular
bacteria provide the basis of specific tests for those
bacteria in a mixture of microorganisms.

5   P.aeruginosa is a bacterium that must be
stipulated as absent in 100 ml of H_2O to be used for
the preparation of pharmaceutical products,
particularly injectables. There is therefore a
considerable need to develop a rapid assay system for

10   this specific bacterium. Existing rapid assays such as
DNA probes or antibody probes cannot discriminate
between live and dead cells, an unacceptable limitation
only avoidable by testing after microbial growth.
Novel processes such as the construction of genetically

15   engineered bacteriophage specific for P.aeruginosa and
containing the luxAB genes are a possibility but cost
of development is high.

          The fact that P.aeruginosa produces a
substance capable of complementing inducer in a luxR/AB

20   construct provides the basis of a rapid test.

          It has been demonstrated that the normal
density dependent expression of the entire lux operon
in E.coli can be suppressed. In liquid growth a lux
operon construct would not emit high levels of light

25   until the culture density approached the end of
exponential growth. If, however, such cells are
immobilised in a low concentration gel the bacteria
will light up very rapidly. It is presumed that this
is a consequence of limiting the diffusion of the

30   autoinducer.

          A concept assay for P.aeruginosa may be as
follows. Water is filtered to collect any bacteria and
the filter overlayed with a gel (agar, agarose or
gelatin) containing E.coli[pSB237]. The inducer

35   produced by live P.aeruginosa cells is limited in
diffusion and rapidly reaches a concentration

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sufficient to activate bioluminescence in the
surrounding E.coli cells. In effect light 'plaques'
appear and can be detected and counted. The ability to
control the optimal media conditions for production of
5   complementing activity allows for considerable
specificity of the assay.

In yet another example of the technique, a
compound according to formula 1 can be added to a
microorganism culture in order to cause expression of a
10 particular gene that would not otherwise be expressed.
For example, the compound may be used to induce
antibiotic production.

In yet another example, growth media for
microorganisms e.g. bacteria can be prepared
15 containing an autoinducer - at an effective
concentration which would lead to a stimulation or
promotion of the metabolism, growth and/or recovery of
the organisms. (These phenomena are herein referred to
collectively as growth.). This may include all the
20 organisms present or, in some cases, it may enhance a
selected group of organisms in a sample in preference
to the others.

A lot of work has been done on the regulation
of the lux operon of Vibrio fischeri, in which the
25 autoinducer of formula 2 plays a crucial role.
Reference is directed to Figure 1 of the accompanying
drawings. The following paragraphs describe the
current state of knowledge regarding the role of
autoinducer in the control of the lux operon and should
30 be read in conjunction with Figure 1.

The phenomenon of autoinduction was first
described by Nealson et al. (1970), based on
observations that Vibrio harveyi and Vibrio fischeri
produce light only at high cell densities, and that
35 th se bacteria produce a substance which will induce
bioluminescence in cultures of low cell density. It

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was postulated that a substance termed ´autoinducer´
accumulates in the growth media and induces the
synthesis of the components of the bioluminescence
system (when its concentration reaches 1-2 molecules
5   per bacterial cell).   Therefore, it is not cell density
per se which influences bioluminescence, but the
accumulation of the autoinducer molecule.   The first
autoinducer molecule to be purified and structurally
identified was that produced by Vibrio fischeri, and
10   was found to be N-(β-ketocaproyl)homoserine lactone
(Eberhard et al., 1981).   It was speculated that as
this molecule combines homoserine lactone, an
intermediate in amino acid metabolism and β-ketocaproic
acid, a relative of fatty acid metabolism
15   intermediates, that it could be a signal of nutritional
viability communicated to other bacteria perhaps to
induce a chemotactic response, hence the autoinducer
became considered a bacterial pheromone (Eberhard,
1972) with a role in population sensing.

20   The luxI gene which lies upstream of luxC in
V.fischeri codes for a 22 kD polypeptide believed to be
responsible for production of autoinducer, apparently
from cytoplasmic precursors (Engebrecht et al., 1983)
although exactly what these precursors are is not
25   clear.   There is no evidence that any systematic search
for genes analogous to luxI in other luminous bacteria
has been attempted.

             In 1987 Kaplan and Greenberg identified a
positive regulatory element in V.fischeri which was
30   required along with autoinducer to activate
transcription of the structural genes for
bioluminescence (luxCDABE) and for autoinducer
synthesis (luxI product).   This regulatory protein was
found to be a 28 kD polypeptid coded for by luxR, a
35   gen which lies upstream of luxI but which is
transcrib d in the opposite direction to the other lux

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genes. The luxR product was shown to be a DNA-binding
protein, although under the conditions used the binding
was not lux DNA-specific. The reason suggested for
this was incorrect renaturation of the protein during
5   purification, an explanation further supported by
problems of reproducibility when trying to demonstrate
binding of autoinducer. Mutational analysis of luxR
has indicated that one region, near the centre of the
luxR polypeptide, constitutes an autoinducer binding
10   domain, while a region towards the carboxy terminus of
the polypeptide constitutes a lux operator DNA
binding/recognition domain (Slock et al., 1990). The
position of the autoinducer binding site has
subsequently been localised between amino acid residues
15   79-127 of the V.fischeri luxR protein (Shadel et al.,
1990). This could, therefore represent a highly
conserved region within luxR homologs.

       More recently, a region unlinked to the lux
structural genes has been identified as a locus
20   controlling bioluminescence in V.harveyi, which, unlike
V.fischeri, does not possess lux regulatory genes as
part of an integral operon required for bioluminescence
(Martin et al., 1989). Nevertheless, there is a region
upstream of luxC in V.harveyi that contains a strong
25   inducable promoter, which is cell density regulated and
glucose repressible (Miyamoto et al., 1990). The
unlinked regulatory locus from V.harveyi (named luxR)
has been cloned and sequenced (Showalter et al., 1990),
and the results indicate a structural relationship to
30   some DNA binding proteins (i.e. the DNA-binding domain
of Cro-like proteins). There is, however, no sequence
similarity to the luxR gene of V.fischeri.
Furthermore, the cloned `luxR´ from V.harveyi is
unresponsive to exogenously supplied autoinducer and
35   does not direct th synthesis of an autoinducer
activity. Th cloning of this regulatory locus into

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E.coli together with luxCDABE does not reconstruct a
lux regulatory system reflective of V.harveyi. It has
been suggested, therefore, that a further missing
function may be identified by the cloning of additional
5   V.harveyi DNA into E.coli which already contains
luxRCDABE on another replicon.

Summary of the control system to date

1.   There is low basal expression of luxI, the
product of which makes autoinducer from cytosolic
10   substrates.

2.   cAMP/CRP activates the luxR promoter and luxR
protein is produced.

3.   LuxR protein interacts with autoinducer to
form a complex (R/AI).

15   4.   R/AI complex binds to lux operator region
between luxR and luxI (perhaps displacing LexA or after
LexA is cleaved due to SOS response).

5.   Binding of R/AI promotes transcription of
right operon (luxICDABE by σ^{32} (htpR protein).

20   Although this invention is based on results
rather than theory, the inventors currently believe
that the compounds of formula 1 form a bacterial
pheromone family which have a primary role in
controlling various functions in bacteria and other
25   microorganisms. These freely soluble and diffusable
low molecular weight molecules may act as sensors of
inter-microbial communication. As density sensors they
may control initiation of maintenance gene expression
(Sigma 32). Sensing isolation they may prevent
30   expression of conjugative gene systems, and again
sensing density, they may trigger the initial Sigma
factor change in bacilli that precedes the sigma
cascade during sporulation.

The following examples illustrate various
35   aspects of th invention.

Example 1 describes a bioassay in which a

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mutant strain of _Erwinia carotovora_ is used to detect
autoinducer, or bacteria which generate autoinducer.
The detectable phenotype in this case is carbapenem
production.

5   Example 2 describes the isolation of N-(β-
ketocaproyl) homoserine lactone from the culture
supernatant of an _Erwinia carotovora_ strain. This is
of interest as a demonstration that a non-
bioluminescent non-marine bacterium produces the
10   autoinducer that controls the _lux_ operon of _Vibrio
fischeri_.

Example 3 describes the preparation of
numerous mutants of the _E.carotovora_ strain which lack
the ability to make carbapenem antibiotics. The
15   example demonstrates that in some of these mutants the
antibiotic production capability can be restored by
addition of exogenous autoinducer.

Example 4 describes a different bioassay for
autoinducer, based on the genetic construct pSB237. A
20   bioassay uses bioluminescence to test for the presence
of autoinducer, or microorganisms that make it. Using
the assay, many bacteria are identified as producers of
the autoinducer.

Example 5 describes the preparation and
25   characterisation of N-(β-ketocaproyl) homoserine
lactone and analogues, both in the racemic form and as
D- and L- isomers.

Example 6 describes a bioassay, along the
lines of Example 1, to detect the analogues described
30 · in Example 5.

Example 7 describes a method for the
enumeration of _Pseudomonas aeruginosa_ using
E.coli[pSB237] as an indicator strain.

Example 8 shows induction of carbapenem
35   production by the L- and D-isomers of autoinducer.

Example 9 describes an experiment to

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demonstrate the action of autoinducer in enhancing the
rate of growth of bacteria.

Example 10 demonstrates activity of various
analogues in inducing luminescence.

Example 11 demonstrates early induction of
carbapenem production by autoinducer in E.carotovora.

Example 12 shows the effect of autoinducer on
the recovery of injured microorganisms.

Example 1

Bioassay for Autoinducer

1.   The bioassay method is based around an
autoinducer non-producing, EMS generated mutant strain
of Erwinia carotovora: B10 002/mu22, henceforth called
by the abbreviated name: mutant 22 (whose preparation
is described in Example 3). The biosynthesis of
carbapenem antibiotic is induced by the autoinducer
compound. Mutant 22 is blocked at some point in the
biosynthesis of autoinducer. Thus, addition of a
sample containing the autoinducer compound will
complement this genetic lesion, allowing the
biosynthesis of antibiotic observed in the strain from
which the mutant is derived.

2.   Samples of up to 50 µl in volume are added to
wells of approximately 10 mm in diameter, cut in Oxoid
DST agar plates seeded with an E.coli carbapenem
super-sensitive strain (E.coli ESS). DST agar is made
up as directed. After autoclaving, the DST agar is
cooled to 45°C, then 3.0 ml per litre of DST agar of a
culture of E.coli ESS added. The latter is grown up
overnight in Oxoid brain heart infusion broth at 37°C.

3.   An inoculum of Mutant 22 is placed around the
rim of the wells containing the samples to be analysed
for autoinducer. Positive and negative controls are
included consisting of: i) antibiotic-producing
strain (Erwinia carotovora ATCC 39048); ii) mutant 22

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with synthetic autoinducer; iii) mutant 22 without
any sample added; iv) and each sample tested for
antibacterial activity in the absence of mutant 22 to
test for presence of antibiotics or residual solvents.

5   4.   The plates are inoculated overnight at 26°C.
If the autoinducer is present in a sample placed in a
well with mutant 22, the antibiotic produced gives a
clearing on the plate.

10   Example 2
Isolation of N-(β-ketocaproyl) homoserine lactone from
the culture supernatant of Erwinia carotovora ATCC39048
            E.carotovora was maintained on nutrient agar
slopes. A loopfull of the culture was inoculated into
15   seed stage medium (consisting of neutralised soya
peptone 1% w/v and sucrose 0.1% w/v) and incubated at
26°C on a rotary shaker for 24 hours. 1 ml was added
to each of four Erlenmeyer flasks, each containing
500 ml production (ECP) medium. This consisted of:

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L-Glutamic acid	0.2% w/v
Ammonium sulphate	0.1% w/v
di-Potassium hydrogen orthophosphate	0.37% w/v
Potassium di-hydrogen orthophosphate	0.62% w/v
Sodium Chloride	0.02% w/v
Casamino acids (Difco)	0.2% w/v
Glucose	0.4% w/v
Ferrous sulphate heptahydrate	0.001% w/v
Magnesium sulphate heptahydrate	0.01% w/v

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            Cultures were incubated on a rotary shaker at
26°C, 220 rpm for 16 hours. Cultures were clarified by
centrifugation (10,000 rpm, 10 minutes), supernatant
taken and extracted twice with 400 ml ethyl acetate
35   (distill d ov r potassium carbonate). The ethyl
acetate layer was taken, 30 ml of distilled water added

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and ethyl acetate removed by rotary evaporation at
35°C.

The aqueous solution thus obtained was passed
twice through a column which contained hydrophobic
5   resin (Styrene-divinylbenzene copolymer CHP3C,
Mitsubishi Ltd.). The column was eluted with distilled
water (60 ml), then 30% v/v methanol in water (60 ml),
then 70% v/v methanol in water (60 ml). Fractions were
monitored throughout for biological activity using the
10   Complementation Bioassay.

The 70% fraction (which contained all
detectable biological activity) was taken and
concentrated to a volume of 3 ml by rotary evaporation
at 35°C. Further substantial purification was achieved
15   by HPLC, set up as follows:

Column   Semi-preparative, Reverse phase
                                (S50DS2, Hi-Chrom Ltd.)
Mobile Phase   15% v/v methanol in water
20   Flow Rate   2 ml/min
Monitoring wavelength   210 nm
Injection volume   0.5 ml

The autoinducer eluted at ca 17.5 minutes.
25   Fractions were pooled, methanol removed by
rotary evaporation at 35°C, aqueous solution freeze
dried to yield 1 mg 99+% pure N-(β-ketocaproyl)
homoserine lactone as a lyophilised white powder.

30   Example 3
Production of Autoinducer non-producing mutants in
Erwinia carotovora ATCC 39048 using ethyl methane
sulphonate (EMS) Mutagenesis
1.   Erwinia carotovora ATCC 39048 is grown in LB
35   medium (Maniatis et al., 1982) containing 10 μg/ml
kanamycin, 50 μg/ml tetracycline in a rotary shaker at

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26°C overnight. OD_{600} is measured to ensure culture
has reached stationary phase ($OD_{600} \geq 2.0$).

2.   Inoculate 1.0 ml of above culture into 50 ml
of fresh complex medium. Place on rotary shaker at
26°C. Grow to $OD_{600} \approx 0.7$.

3.   Centrifuge 1.0 ml of culture in a sterile
microfuge tube at 12 K rpm for 2 minutes at room
temperature. Resuspend cells in 1.0 ml of sterile SPC
buffer (SPC buffer: 0.15 M NaCl, 10.8 mM NaH_2PO_4,
9.0 mM citric acid (pH 7.0)). Cells were pelleted as
above, supernatant discarded and cells resuspended in
1.0 ml of sterile SPC buffer.

4.   25 µl of ethyl methane sulphonate (EMS) is
added to 1.0 ml of cells suspended in SPC buffer, to
give a 2.5% solution of EMS. Cells are then incubated
with EMS without shaking for 1 hour at room temperature.

5.   Centrifuge cells in a microfuge at 12 k rpm
for 2 mins. Remove supernatant and resuspend cells in
1.0 ml of sterile 5% sodium thiosulphate (pH 7.0).
Wash the cells with 1.0 ml of sterile SPC buffer twice
to remove any traces of sodium thiosulphate.

6.   Resuspend cells in 1.0 ml LB medium. Place
in 26°C incubator without shaking for 1 hour.

7.   Dilutions of cells are made in sterile water
and 100µl volumes spread on nutrient agar plates
containing 10 µg/ml kanamycin, 50 µg/ml tetracycline.
Plates are incubated at 26°C for 48 hours.

8.   Colonies were picked-off the above plates
using sterile toothpicks and an inoculum placed on DST
agar plates seeded with E.coli ESS strain (Oxoid DST
agar made up as directed. After autoclaving, agar is
cooled to 45°C and 3.0 ml of an overnight culture of
E.coli ESS strain in Oxoid brain heart infusion broth
added to 1 litre of DST). Plates are incubated at 26°C
for 24 hours. When carbapenem antibiotic is produced by
the Erwinia colony a cl aring is observed. Non-

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producers of carbapenem antibiotic are selected.
Amongst those are autoinducer non-producing mutant
colonies (about 20% of the total showing a phenotype of
antibiotic non-production).

5   9.   Autoinducer non-producing mutant strains have
been identified by complementation analysis using
whole cells, filter sterilised culture supernatants and
HPLC analysis of culture supernatants.

10   <u>Complementation Test of Mutants</u>
     1.   Using Oxoid DST agar seeded with <u>E.coli</u>
super-sensitive strain (ESS), it can be shown that some
mutants derived from <u>Erwinia</u> <u>carotovora</u> ATCC 39048 are
no longer able to synthesise carbapenem antibiotic.

15   Amongst these are mutants unable to synthesise
autoinducer which is a requirement for carbapenem
biosynthesis. Due to the diffusable nature of the
autoinducer molecule a mutant defective in the
biosynthesis of the compound will be complemented by

20   strains with mutations in the carbapenem biosynthetic
pathway or regulatory autoinducer binding proteins,
which will have the functional autoinducer biosynthetic
machinery.
     2.   Complementation is observed by mixing whole

25   cells of mutant strain B10 002/mutant 22 (subsequently
called mutant 22) with other mutants such as mutant
strain B11 001/mutant 26 (subsequently called mutant
26).
     3.   Mutants are mixed together on DST agar seeded

30   with <u>E.coli</u> ESS. When mixed, production of antibiotic
and concomitant clear zone on the plate are observed.
Separate inoculums of each mutant do not produce any
antibiotic.
     4.   The same effect can be observed when feeding

35   filter-sterilised sup rnatant from a culture of one
mutant to a mutant of a separate complementation group.

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A 50 µl sample is placed in a well cut in a DST agar
plate seeded with E.coli ESS, with the chosen mutant
inoculated around the rim of the well. The plate is
incubated at 26˚C for 24 hours, after which time clear
5   zones produced by the presence of antibiotic will be
visible.

5.   Feeding the supernatant from a mutant such as
mutant 26 to mutant 22 induces antibiotic production in
the latter. No induction of carbapenem is observed
10 when filter-sterilised supernatant from mutant 22 is
incubated with mutant 26 on bioassay plates.

The following Table lists the mutant strains
prepared.

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Table 1

Compilation of Erwinia carotovora strains with antibiotic non-producing phenotypes

COMPLEMENTATION GROUP 1			COMPLEMENTATION GROUP 2		
strain	number	mutagen used	strain	number	mutagen used
A05 002	8	NTG	B03 001	4	EMS
A05 003	9	NTG	B10 002	22	EMS
A05 005	10	NTG	B10 003	23	EMS
A05 020	11	NTG			
A05 026	12	NTG			
A06 001	13	NTG			
A07 005	14	NTG			
A07 037	15	NTG			
A08 003	16	NTG			
A08 004	17	NTG			
A08 017	18	NTG			
A08 020	19	NTG			
A08 022	20	NTG			
B10 001	21	EMS			
B10 004	24	EMS			
B10 005	25	EMS			
B11 001	26	EMS			

The first three digits of the mutant strain number indicate individual mutagenesis experiments.

	COMPLEMENTATION GROUP 1	COMPLEMENTATION GROUP 2
Total number of mutants produced	17	3

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Example 4
The construction of pSB 237: a V.fischeri autoinducer sensor controlling a bioluminescent phenotype in E.coli

5   An E.coli vector pSB226 containing a promoterless copy of the luxA and luxB genes from V.harveyi has been previously described (Hill et al., 1991)

        This vector has a unique EcoR1 site proximal

10   to luxA which facilitates the insertion of promoterless elements. A luxR lux promoter region of the V.fischeri lux operon was obtained by PCR using the following primers designed to incorporate terminal EcoR1 sites and to have homology to sequences previously identified

15   by Engebrecht and Silverman (1987).

Primer (1) (5´ end of luxR)
5´   3´

AAG CTT GAATTC CCG GGT TAA TTT TTA AAG TAT GGG CAA TCA ATT

20

Primer (2) (3´ end of lux promoter)
5´   3´C

TTT TAT GAATTC TAC GTA ACC AAC CTC CCT TGC GTT TAT TCG A
Recognition sequences for EcoR1 are underlined.

25   The PCR fragment obtained by use of the above primers was digested with EcoR1 and inserted into the unique EcoR1 site of pSB226. Recombinant clones were selected on the basis of a bioluminescent phenotype dependent upon the presence of dodecanal vapour and

30   synthetic V.fischeri autoinducer. One such recombinant was designated pSB237.

        This example uses E.coli [pSB237], a bacterium in which, as noted above, bioluminescence is expressed only in the presence of exog nous N-(β-

35   ketocaproyl) homoserine lactone (or analogue). This phenotype is used as a basis of a test for synthetic

- 20 -

autoinducer or for other bacteria that may produce
autoinducer.

Bioluminescent bioassay for autoinducer
5   The bioassay method is based on E.coli
[pSB237], described above. Overnight LB cultures
(Maniatis et al., 1982) of E.coli [pSB237] are used to
initiate exponential cultures by appropriate dilution
into fresh LB media. 100 μl of exponential cultures
10   are placed into microtitre wells within a microtitre
tray format. 100 μl of cell free culture supernatant
or appropriately diluted N-(β-ketocaproyl) homoserine
lactone is added individually to wells so that the
potential to induce bioluminescence from E.coli
15   [pSB237] can be assessed. Microtitre trays are
incubated at 30°C prior to bioluminescence
determination which must be preceded by the addition to
each well of 5 μl of a 1% dodecanal solution in
ethanol. The limits for detection of autoinducer
20   using a bioluminescent phenotype derived from E.coli
[pSB237] depend upon the time of exposure of the
bacterial culture to inducer. At concentrations above
10 ng/ml of culture, induction of bioluminescence is
rapid (less than 10 min). At lower concentrations
25   induction is progressively slower but concentrations as
low as 80 pg/ml can be distinguished from a zero
concentration control after some 20 h of incubation
(Figure 2). This establishes the bioluminescent assay
as a highly sensitive and simple monitor of inducer.

30

Pseudomonas aeruginosa
           P.aeruginosa produces complementing activity
(confirmed as autoinducer) only under defined growth
conditions. In rich media such as LB or Brain Heart
35   Infusion Broth the degre of complem nting activity is
low. In limiting or minimal growth m dia and

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particularly when supplemented with 1 mg/ml Fe(III),
the production of complementing activity is very
considerable. By comparison with standard
concentrations of autoinducer (L-isomer), the

5   supernatant of such a _P.aeruginosa_ culture could be
estimated to contain some 10 ng/ml of autoinducer. The
media dependency of complementation activity strongly
suggests that production is regulated and that under
favourable conditions production levels may equate to

10   those previously reported in marine bacteria (Eberhard,
1981; 1 µg/ml).

Serratia marcescens

S. marcescens is capable of providing a
15   similar level of complementing activity for
E.coli[pSB237] as _P.aeruginosa_. the major difference
is that in this instance there is no media variability
with very high complementations achieved from BHI
supernatants. This would suggest constitutive
20   expression of autoinducer, a feature previously
described for _V.fischeri_ (Eberhard _et al_., 1981).

Proteus mirabilis and Citrobacter freundii

Both of these bacteria produce complementing
25   activity in a limiting growth medium (CCY; Stewart _et
al_., 1981). The activity detailed by the
bioluminescent assay indicates a lower level of
inducer formation or, alternatively, production of an
inducer analogue with lower activity for the luciferase
30   _lux_R product. Nevertheless, the rate of appearance of
activity would reflect inducer levels of greater than 1
ng/ml for a homologous compound.

35

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Microorganisms with low but detectable activities

      Table 2 and the accompanying Figure 3 list
microorganisms that provide complementation for
activation of bioluminescence with E.coli[pSB237].

5   Typically, activity can take between 0.5 and 6 hours to
become measurable above background controls. Given the
dependence upon time for detection of low levels of
autoinducer, it is possible that these long incubation
periods reflect low levels of inducer in the microbial

10   cultures. It is particularly interesting to note that
almost none of the cultures provided any complementing
activity in rich media but that the limiting CCY media
was particularly productive.

      It is possible that the production of inducer

15   analogues of low intrinsic activity with E.coli
[pSB237] could be an alternative explanation of the
above but equally these results could reflect the
detection of basal levels of autoinducer production.
These could, after appropriate physiological trigger

20   events, be switched to high production for gene control
functions.

      Other tests have shown positive results (i.e.
production of autoinducer) also by the following
microorganisms: Hafnia alvei, Serratia liquefaciens,

25   Enterobacter agglomerans, and Rahnella aquatilis.

30

35

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Table 2

A_1	Aeromonas hydrophila		A_2	Citrobacter freundii 6071
B_1	CONTROL		B_2	Citrobacter freundii 9756
C_1	Bacillus cereus		C_2	Citrobacter koseri 10849
D_1	Bacillus coagulans		D_2	Bacillus coagulans
E_1	Bacillus megaterium		E_2	Bacillus cereus 866
F_1	Bacillus megaterium KM		F_2	Bacillus licheniformis
G_1	Bacillus subtilis		G_2	Bacillus macerans
H_1	Brochothrix thermosphacta		H_2	Bacillus megaterium 9885

A_3	Bacillus pumilis		A_4	Alcaligenes faecalis
B_3	Bacillus subtilis 2129		B_4	Klebsiella aerogenes
C_3	Bacillus subtilis var niger		C_4	Listeria grayii
D_3	Bacillus globigii		D_4	Listeria monocytogenes 4859
E_3	Escherichia coli 86		E_4	Listeria monocytogenes 23074
F_3	Enterobacter aerogenes		F_4	Listeria monocytogenes 5348
G_3	Enterobacter cloacae		G_4	Micrococcus luteus
H_3	Erwinia herbicola		H_4	CONTROL

A_5	Proteus mirabilis		A_6	Pseudomonas putida JT1
B_5	Proteus vulgaris		B_6	Salmonella arizonae
C_5	CONTROL		C_6	Salmonella infantis
D_5	Pseudomonas aeruginosa PAO1 (KILLED)		D_6	Salmonella montevideo
E_5	Pseudomonas fluorescens		E_6	Salmonella typhimurium LT2
F_5	Pseudomonas putidas		F_6	Serratia marcescens (KILLED)
G_5	Pseudomonas putida 340		G_6	Streptococcus faecalis
H_5	Pseudomonas putida IC4A		H_6	Streptococcus pyogenes

A_7	Streptococcus mutans
B_7	CONTROL
C_7	CONTROL
D_7	Staphylococcus aureus
E_7	Vibrio cholerae non 01
F_7	Escherichia coli K12
G_7	5 ng autoinducer (KILLED)
H_7	-----

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Example 5

Synthesis of the Complementation Factor (Autoinducer)
[N-(3-oxohexanoyl)-L-homoserine lactone] and its analogues

1. Synthesis of N-(3-oxoalkanoyl)homoserine lactones
     (Compounds No. 1, 2, 3, 4, 14, 15 and 16)

General Method

          Triethylamine (1 mmol) was added to a stirred
solution of homoserine lactone hydrochloride (the L- or
D- isomer or a racemic mixture) (1 mmol) in water
(2 ml) followed by the addition of ethylene glycol
ketal of 3-oxoalkanoic acid (1 mmol) and 1-ethyl-3-(3-
dimethylaminopropyl)carbodiimide hydrochloride
(1 mmol). The mixture was stirred for 20 h and then
rotary evaporated to dryness at about 35°C. The light
orange residue was extracted with warm ethyl acetate (5
x 5 ml) and the extracts pooled and washed successively
with water (1 x 3 ml), 5% sodium bicarbonate solution
(1 x 3 ml), 1 M potassium hydrogen sulphate solution (1
x 3 ml) and finally brine (1 x 5 ml). Drying (MgSO$_4$)
and evaporation of solvent in vacuo gave the ethylene
glycol ketal of 3-oxoalkanoylated homoserine lactones
(40-50%).

          Perchloric acid (60%, 0.25 ml) was added to
an ice-cooled solution of the alkanoylated lactone
(0.5 mmol) in dichloromethane (15 ml). The mixture was
stirred at 0°C for 0.5 h and then at room temperature
for 1.5 h. The solvent was removed in vacuo and the
residue redissolved in ethyl acetate (20 ml). The
solution was washed with cold water (2 x 5 ml) and
brine (1 x 5 ml), dried (MgSO$_4$) and rotary evaporated
to obtain the desired N-(3-oxoalkanoyl)homoserine
lactones (55-60%).

          Compound 16 is derived from homocysteine
lactone instead of homoserine lactone.

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2.   Synthesis of N-acylated homoserine lactone
     (Compounds No. 5, 6, 7, 8 and 9)
     General Method

     Triethylamine (1 mmol) was added to a stirred

5   solution of homoserine lactone hydrochloride (the L- or
    D-isomer or a racemic mixture) (1 mmol) in water
    (2 ml) followed either by the addition of acid
    anhydride (3 mmol) (compounds 5, 6 and 7) or acid
    (1.5 mmol) and 1-ethyl-3-(3-

10 dimethylaminopropyl)carbodiimide hydrochloride
    (1.5 mmol) (compounds 8 and 9) . The mixture was
    stirred at room temperature overnight and then
    evaporated in vacuo to dryness. The residue was
    partitioned between water (5 ml) and ethyl acetate (20

15 ml) and the organic layer successively washed with 5%
    NaHCO$_3$ solution (2 x 5 ml), 1 M KHSO$_4$ solution (1 x 5
    ml) and brine (1 x 5 ml). Drying (MgSO$_4$) and removal
    of solvent gave the title acylated lactones (20-60%).

20 3.   Synthesis of N-(3-hydroxyalkanoyl)-L-homoserine
         lactones (Compounds 10, 11, 12 and 13)
         General Method

     N-(3-Oxoalkanoyl)-L-homoserine lactone
    (0.2 mmol) was dissolved in methanol (5 ml) and the

25 solution made acidic (pH 3-4) with 2 M HCl-methanol.
    Sodium cyanoborohydride (0.5 mmol) was added in one lot
    with stirring and the reaction mixture maintained at pH
    3-4 by the occasional addition of 2 M HCl-methanol.
    After 2 h, solvent was removed in vacuo and ethyl

30 acetate extracts (3 x 5 ml) of the residue were
    combined, dried (MgSO$_4$) and evaporated to yield the
    title hydroxy derivatives. The products were purified
    by preparative layer chromatography on silica plates in
    CHCl$_3$-MeOH (9:1) and repurified by HPLC. The latter

35 also resolved and separated the diastereoisomers in the
    case of compounds 10 and 11.

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Autoinducer and its analogues prepared by
these methods were more than 90% pure and were further
purified with reverse phase HPLC using a 1 x 25 cm
S50DS2 semi-prep column eluting isocratically with 15-
20% MeOH-H_2O mixture and monitoring at 210 nm. The
products were freeze-dried and stored below 0°C.

All the compounds (see Table 3) were
characterised by i.r., mass spectra (EI) and high field
n.m.r. as follows.

Compound 1 [N-(3-oxohexanoyl)-L-homoserine lactone]

ν max (KBr) 3295 (NH), 1780 (ring C=O), 1710
ketone C=O), 1650 (amide C=O), 1550, 1170 cm^{-1}.

m/z (EI) (%) 213.0989 (47, M^+, $C_{10}H_{15}NO_4$
requires m/z 213.1001), 185 (13), 170 (8), 155 (7), 143
(33), 128 (8), 113 (19), 102 (56), 101 (35), 71 (82),
57 (100).

δ_H (CDCl$_3$) (400 MHz) 0.9 (3H, t, CH$_3$), 1.64
(2H, sextet, CH$_3$.CH$_2$), 2.22 (1H, dddd, 4α-H), 2.51 (2H,
t, CH$_2$.CO), 2.77 (1H, ddd, 4β-H0, 3.47 (2H, s,
CO.CH$_2$.CO), 4.28 (1H, ddd, 5α-H), 4.48 (1H, dd, 5β-H),
4.59 (1H, ddd, 3-H), 7.65 (1H, bs, NH).

δ_{13C} (CDCl$_3$) 13.58 (CH$_3$), 16.91 (CH$_2$), 29.94
(CH$_2$), 45.85 (CH$_2$), 28.13 (CH$_2$), 49.12 (CH), 65.94
(CH$_2$), 166.38 (CO.NH), 174.83 (ring C=O), 206.54 (C=O).

Compound 3 [N-(3-oxopentanoyl)-L-homoserine lactone]

ν max (KBr) 3280 (NH), 1780 (ring C=O), 1710
(ketone C=O), 1645 (amide C=O), 1550, 1170 cm^{-1}.

m/z (EI) (%) 199.0845 (25, M^+, $C_9H_{13}NO_4$
requires m/z 199.0845), 170 (9), 154 (7), 141 (6), 125
(7), 102 (34), 101 (34), 57 (100), 43 (36).

δ_H (CDCl$_3$) (400 MHz) 1.09 (3H, t, CH$_3$), 2.23
(1H, dddd, 4α-H), 2.57 (2H, q, CH$_3$.CH$_2$), 2.77 (1H, ddd,
4β-H), 3.48 (2H, s, CO.CH$_2$.CO), 4.28 (1H, ddd, 5α-H),
4.48 (1H, dd, 5β-H), 4.58 (1H, ddd, 3-H), 7.60 (1H, bs, NH).

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Compound 4 [N-(3-oxobutanoyl)homoserine lactone]

γ max (KBr) 3280 (NH), 1780 (ring C=O), 1710 (ketone C=O), 1640 (amide C=O), 1550, 1170 cm^{-1}.

m/z (EI) (%) 185.0701 (3, M$^+$, $C_8H_{11}NO_4$

5   requires m/z 185.0687), 140 (4), 127 (7), 102 (4), 101 (21), 57 (72), 43 (100).

δ_H (CDCl$_3$) (400 MHz) 2.23 (1H, dddd, 4α-H), 2.28 (3H, s, CH$_3$), 2.77 (1H, ddd, 4β-H), 3.50 (2H, s, CO.CH$_2$.CO), 4.28 (1H, ddd, 5α-H), 4.48 (1H, dd, 5β-H),

10   4.59 (1H, ddd, 3-H), 7.60 (1H, bs, NH).

Compound 6 [N-butanoyl-L-homoserine lactone]

γ max (KBr) 3310 (NH), 1775 (ring C=O), 1640 (amide C=O), 1545, 1175 cm^{-1}.

15   m/z (EI) (%) 171.0922 (12, M$^+$, $C_8H_{13}NO_3$ requires m/z 171.0896), 153 (4), 143 (65), 128 (5), 125 (5), 102 (10), 101 (10), 71 (57), 57 (65), 43 (100).

δ_H (CDCl$_3$) (400 MHz) 0.96 (3H, t, CH$_3$), 1.68

20   (2H, sextet, CH$_3$.CH$_2$), 2.19 (1H, dddd, 4α-H), 2.24 (2H, t, CH$_2$.CO), 2.82 (1H, ddd, 4β-H), 4.29 (1H, ddd, 5α-H), 4.47 (1H, dd, 5β-H), 4.59 (1H, ddd, 3-H), 6.26 (1H, bs, NH).

25   Compound 7 [N-acetyl-L-homoserine lactone]

γ max (KBr) 3300 (NH), 1785 (ring C=O), 1640 (amide C=O), 1535, 1185 cm^{-1}.

m/z (EI) (%) 143.0546 (6, M$^+$, $C_6H_9NO_3$ requires 143.0582), 125 (5), 116 (2), 101 (3), 98 (11),

30   57 (93), 43 (100).

δ_H (CDCl$_3$) (400 MHz) 2.06 (3H, s, CH$_3$), 2.19 (1H, dddd, 4α-H), 2.78 (1H, ddd, 4β-H), 4.29 (1H, ddd, 5α-H), 4.47 (1H, dd, 5β-H), 4.62 (1H, ddd, 3-H), 6.57 (1H, bs, NH).

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Compound 8 {N-[(E)-hex-2-enoyl]-L-homoserine lactone}

ν_{max} (KBr) 3310 (NH), 1780 (ring C=O), 1675 (amide C=O), 1635 (C=C), 1555, 1175 cm^{-1}.

m/z (EI) (%) 197.1088 (11, M$^+$, $C_{10}H_{15}NO_3$

5   requires m/z 197.1052), 154 (9), 97 (100), 85 (3).

δ_H (CDCl$_3$) (400 MHz) 0.94 (3H, t, CH$_3$), 1.48 (2H, sextet, CH$_3$.CH$_2$), 2.17 (3H, m. 4α-H and CH$_2$.CH=CH), 2.86 (1H, m, 4β-H), 4.31 (1H, ddd, 5α-H), 4.48 (1H, dd, 5β-H), 4.63 (1H, ddd, 3-H), 5.83 (1H, dt,

10   CH=CH.CO), 6.11 (1H, d, NH), 6.90 (1H, dt, CH=CH.CO).

Compound 9 [N-hexanoyl-L-homoserine lactone]

ν_{max} (KBr) 3315 (NH), 1775 (ring C=O), 1645 (amide C=O), 1550, 1175 cm^{-1}.

15   m/z (EI) (%) 199.1256 (5, M$^+$, $C_{10}H_{17}NO_3$ requires m/z 199.1208), 170 (5), 156 (8), 143 (100), 85 (10).

δ_H (CDCl$_3$) (90 MHz) 0.87 (3H, t, CH$_3$), 1.0-1.40 (4H, m, CH$_3$.CH$_2$.CH$_2$), 1.40-1.85 (2H, m,

20   CH$_2$.CH$_2$.CO), 1.85-2.45 (3H, m, CH$_2$.CO and 4α-H), 2.45-2.90 (1H, m, 4β-H), 4.0-4.75 (3H, m, 5-H$_2$ and 3-H), 6.43 (1H, d, NH).

Compound 10 {N-[(S)-3-hydorxyhexanoyl]-L-homoserine

25   lactone}

m/z (EI) (%) 197.0995 (9, M$^+$-H$_2$O, $C_{10}H_{15}NO_3$ requires m/z 197.1052), 172.0629 (52, M$^+$-C$_3$H$_7$, $C_7H_{10}NO_4$ requires m/z 172.0610), 154 (4), 143 (31), 102 (100).

δ_H (CDCl$_3$) (400 MHz) 0.94 (3H, t, CH$_3$), 1.44

30   (2H, m, CH$_3$.CH$_2$), 1.55 (2H, m, CH$_2$.CH.OH), 2.18 (1H, dddd, 4α-H), 2.35 (1H, dd, CH$_\alpha$H$_\beta$.CO), 2.44 (1H, dd, CH$_\alpha$H$_\beta$.CO), 2.83 (1H, dddd, 4β-H), 3.07 (1H, br s, OH), 4.04 (1H, dddd, CHOH), 4.28 (1H, ddd, 5α-H), 4.48 (1H, ddd, 5β-H), 4.57 (1H, ddd, 3-H), 6.55 (1H, br s, NH).

35

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<u>Compound 11</u> ($\underline{N}$-[(R)-3-hydroxyhexanoyl]-L-homoserine lactone)

m/z (EI) (%) 197.1066 (11, M^+-H_2O, $C_{10}H_{15}NO_3$ requires m/z 197.1052), 172.0612 (53, M^+-C_3H_7, $C_7H_{10}NO_4$
5   requires m/z 172.0610), 143 (27), 102 (100).

δ_H ($CDCl_3$) (400 MHz) 0.94 (3H, t, CH_3), 1.43 (2H, m, $CH_3.C\underline{H}_2$), 1.55 (2H, m, $C\underline{H}_2.CH.OH$), 2.19 (1H, dddd, 4α-H), 2.33 (1H, dd, $C\underline{H}_\alpha H_\beta CO$), 2.46 (1H, dd, $CH_\alpha \underline{H}_\beta.CO$), 2.83 (1H, dddd, 4$\beta$-H), 4.05 (1H, dddd,
10 $C\underline{H}.OH$), 4.30 (1H, ddd, 5α-H), 4.48 (1H, ddd, 5β-H), 4.55 (1H, ddd, 3-H), 6.50 (1H, br s, NH).

<u>Compound 12</u> (N-[(RS)-3-hydroxypentanoyl]-L-homoserine lactone)

15 m/z (EI) (%) 201.0939 (2, M^+, C_9H_{15}-NO_4 requires m/z 201.1001), 183.0931 (8, M^+-H_2O, $C_9H_{13}NO_2$ requires m/z 183.0895), 172.0677 (24, M^+-C_2H_5, $C_7H_{10}NO_4$ requires m/z 172.0610), 143 (28), 102 (44), 101 (32), 57 (100).

20 δ_H ($CDCl_3$) (90 MHz) 0.93 (3H, t, CH_3), 1.50 (2H, quintet, $CH_3.C\underline{H}_2$), 2.15 (1H, m, 4α-H), 2.33 (2H, d, $CH_2.CO$), 2.60 (1H, m, 4β-H), 2.70 (1H, br s, D_2O exchangeable, OH), 4.0-4.80 (3H, m, 5-H_2 and 3-H), 6.90 (1H, br s, D_2O exchangeable, NH).

25

<u>Compound 13</u> ($\underline{N}$-[(RS)-3-hydroxybutyryl]-L-homoserine lactone)

m/z (EI) (%) 187.0820 (2, M^+, $C_8H_{13}NO_4$ requires m/z 187.0844), 172.0614 (11, M^+-CH_3, $C_7H_{10}NO_4$
30 . requires m/z 172.0610), 169.0761 (6, M^+-H_2O, $C_8H_{11}NO_3$ requires m/z 169.0739), 143 (34), 102 (46), 101 (18), 57 (100).

δ_H (D_2O) (90 MHz) 1.20 (3H, d, CH_3), 2.20 (1H, m, 4α-H), 2.40 (2H, d, $C\underline{H}_2.CO$), 2.57 (1H, m, 4α-
35 H), 4.0-4.70 (4H, m, 5-H_2, 3-H and $C\underline{H}.OH$).

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Compound 14 [N-benzoylacetyl-L-homoserine lactone]

     m/z (EI) (%) 247.0824 (24, M^+, $C_{13}H_{13}NO_4$
requires m/z 247.0844), 147 (12), 105 (100), 77 (37).

     δ_H (CDCl$_3$/DMSO-d$_6$) (90 MHz) 2.20-2.80 (2H,
m, 4-H$_2$), 3.94 (2H, s, CO.CH$_2$.CO), 4.10-4.80 (3H, m,
5-H$_2$ and 3-H), 7.30-7.70 (3H, m, ArH), 7.95 (2H, dd,
ArH), 8.56 (1H, d, NH).

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Table 3

Autoinducer and its analogues
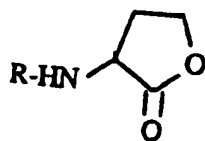
No.	R	Chirality at C-3	Overall Yield from Homoserine Lactone
1	$CH_3.CH_2.CH_2.CO.CH_2.CO$	L	25%
2	$CH_3.CH_2.CH_2.CO.CH_2.CO$	D	26%
3	$CH_3.CH_2.CO.CH_2.CO$	L	28%
4	$CH_3.CO.CH_2.CO$	D,L	28%
5	$CH_3.CH_2.CH_2.CO$	D,L	39%
6	$CH_3.CH_2.CH_2.CO$	L	40%
7	$CH_3.CO$	L	20%
8	$CH_3.CH_2.CH_2.CH=CH.CO$	L	35%
9	$CH_3.CH_2.CH_2.CH_2.CH_2.CO$	L	60%
10	$(S)-CH_3.CH_2.CH_2.CH(OH).CH_2.CO$	L	5%
11	$(R)-CH_3.CH_2.CH_2.CH(OH).CH_2.CO$	L	5%
12	$(RS)-CH_3.CH_2.CH(OH).CH_2.CO$	L	8%
13	$(RS)-CH_3.CH(OH).CH_2.CO$	L	7%
14	$Ph.CO.CH_2.CO$	L	20%
15	$CH_3.CH_2.CH_2.CH_2.CO.CH_2.CO$		
16*	$CH_3.CH_2.CH_2.CO.CH_2.CO$		

* In this compound the ring oxygen atom is replaced by sulphur

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Example 6

Erwinia mutant PNP22 was inoculated around
the rims of 3 mm wells cut into agar plates seeded with
E.coli ESS. Solutions of a range of concentrations of
5   each analogue of Example 5 were prepared in distilled
water, filter sterilised and 50 μl added to each well.
Plates were incubated at 26°C overnight and the
resulting inhibition zone diameters measured (mm).
Figure 4 is a graph of inhibition zone diameter (which
10   denotes induction of carbapenem antibiotic production)
against concentration of various compounds. The dose
response curves are shown for compounds 1, 14, 3, 11,
12, 9, 10 and 6. The remaining compounds in the series
showed little or no activity in this assay, apart from
15   the D isomer which shows about 10% activity (data not
shown).

Example 7
The enumeration of Pseudomonas aeruginosa using
20   E.coli[pSB237] as an indicator strain.

A method for the enumeration of Pseudomonas
aeruginosa was investigated using E.coli[pSB237]
(Figure 5) as an indicator. pSB237 confers an
autoinducer-dependent bioluminescent phenotype on
25   E.coli (upon addition of exogenous aldehyde) and can
therefore detect the production of autoinducer from
P. aeruginosa. The experiment requires an over-night
culture of P. aeruginosa which is subjected to 10 fold
serial dilutions such that approximately 2, 20 and 200
30   colonies are filtered onto a nitrocellulose membrane.
We have found the use of membranes to be preferable to
pour plate methods because the membranes provides an
aerobic environment for the Pseudomonas to grow. The
membranes are placed onto a lawn of E. coli[pSB237].
35   The inducer from P. aeruginosa diffuses through the
membrane and triggers bioluminescence in localised

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areas of the E. coli[pSB237] lawn equivalent in size to
the Pseudomonas colony. The number of light areas
exactly correlate with the number of Pseudomonas
colonies. In a mixed culture only those bacterial

5   colonies which produce autoinducer will provide light
in the E. coli[pSB237] lawn and this can, therefore,
provide a very clear discrimination between what may
otherwise be closely related bacteria.

10   Experimental details
1.   Overnight cultures of P. aeruginosa PAO1 and
E. coli[pSB237] were grown in Luria Broth.
2.   A 10 fold dilution series of the overnight
Pseudomonas culture was prepared using Luria Broth and

15   samples plated onto Luria Broth Agar to obtain colony
counts.
3.   0.5 ml of the overnight E. coli[pSB237] was
added to 4 ml of molten (45°C) Luria Broth Agar and
spread onto a Luria Broth Agar plate.

20   4.   1 ml of the Pseudomonas dilution series -8,
-7 and -6 (in duplicate) were individually filtered
onto a Gelman 0.45 µm nitrocellulose membrane. These
were subsequently individually placed onto
E. coli[pSB2237] agar lawns as prepared in (3) above.

25   5.   Plates were incubated at 30°C for several
hours and viewed with the Hamamatsu Argus 100 Vim 3
camera after exposure to nonanal vapour.

Results

30

Dilution	No. of Light Areas	No. of colonies
-6	Too many to count	Too many to count
-7	170/168	158/182
-8	23/22	21/22

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Example 8

Induction of carbapenem biosynthesis in liquid culture

A culture of mutant PNP22 was grown overnight in ECP medium and a 2% inoculum (v/v) added to 100 ml of fresh ECP medium. This was incubated on a rotary shaker (rpm 220) at 26°C for 6 hours (until OD > 2.0).

Carbapenem HPLC assay

Bacteria were harvested by centrifugation at 10000 rpm for 10 mins. The supernatant was extracted with a solution of 4% Aliquat 336 (Aldrich) in distilled dichloromethane. Samples were analysed by HPLC using a reverse phase, semi-preparative column.

Induction of carbapenem biosynthesis in PNP22 by addition of exogenous autoregulator

Synthetically produced autoregulator was added at a range of concentrations from 0 to 75 µg/ml and a dose-response curve constructed. The threshold for induction of carbapenem production is approximately 0.5 µg/ml. The optimum concentration is 1.0 µg/ml. Above this concentration, biosynthesis of carbapenem appears to be inhibited.

The experiment was repeated using the D-isomer in place of the L-isomer (i.e. compound 2 from Example 5 in place of compound 1). In this case the threshold for induction of carbapenem production was about 2 µg/ml and the optimum concentration was 30 µg/ml.

Example 9

Effect of Autoinducer in microbial media on promoting microorganism metabolism

The measurement of microorganism metabolism can be monitored by several methods one of which is known as imp dance monitoring. It has be n shown that when bacteria grow in culture media the end products of metabolism are generally more highly charged than the

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original growth substrates. The resulting changes in
impedance can be monitored and "growth" or metabolism
curves constructed. The curves obtained by growth in
different substrates can be compared for the growth
5   quality of the substrates. The curves can also be used
to determine the number of bacteria in a given sample,
estimate the sensitivity of microorganisms to
inhibitory substances such as antibiotics and also
determine the utilisation of growth substances.
10

Experimental

     Experiments were performed to examine the
effects of autoinducer in culture media on the curves
obtained using a Malthus AT growth analyser.
15   Serial tenfold dilution of a 4 hour culture
in nutrient broth (Lab M, lab 14) of Serratia
marcescens were prepared in sterile 0.9% saline.
0.02 ml of 10^{-4} or 10^{-5} dilution were added to 6 tubes
of media as detailed below. The tubes were mixed and
20   then attached to the Malthus system.
     Easter & Gibson media (Lab M, lab 137) was
used throughout with 5 g per litre tri-methyl amine N-
oxide added before autoclaving. Sodium biselenite was
omitted and the medium was prepared according to the
25   manufacturer's instructions. The inducer (a synthetic
preparation of N-(β-ketocaproyl) homoserine lactone)
solution was prepared in sterile water and added to the
Malthus tubes to give varying final concentrations up
to 80 ng/ml.
30   8 of 9 sets of experiments showed an increase
of maximum output of between 300 - 500 microsiemens at
concentrations of between 5 and 20 ng/ml autoinducer
(as compared to the output at zero autoinducer). These
results clearly demonstrate that increased bacterial
35   growth was obtained in the presence of inducer.

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Example 10

Activity of various analogues in inducing luminescence

Autoinducer and various analogues described
in Table 3 were tested for their ability to induce
luminescence in E.coli[pSB237] in the assay described
in Example 4.

Compound	Light Levels
1	+++
3	++
12	++
6	+
8	+

In another experiment, tenfold dilutions of
autoinducer and various analogues were tested for their
ability to induce luminescence in E. coli[psB237]. The
following results were obtained, expressed as light
levels as above:-

Dilution	Compound		
	1	15	16
-2	++++	+++	++++
-3	++++	+++	++++
-4	+++	++++	++++
-5	+	++++	+
-6	-	++++	-

Example 11

Early Induction of Carbapenem Production by Autoinducer
in Erwinia carotovora ATCC 39048

Erwinia carotovora ATCC 39048 was grown as in
Example 2, except that 1 ml of overnight seed culture
was inoculated into 50 ml ECP with and without
autoinducer at a final concentration of 5 µg/ml. The

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cultures were incubated at 26°C on a shaking incubator
at 240 rpm. 100 μl samples of supernatant were taken
and injected onto a Spherisorb S5ODS2 HPLC column
eluted with 100 mM KH_2PO_4 at 2 ml/min. The carbapenem
5   peak eluted at approximately 7.8 minutes and was
determined by absorbance at 254 nM. Inclusion of
autoinducer switched on carbapenem production (3-6 hr)
several hours earlier than control cultures (5-8 hr).

10   Example 12
Effects on recovery of injured organisms

Aim
To test the effect of Autoinducer on recovery
15   on selective and non-selective agar of Serratia
marcescens bacteria with freezing induced injuries.

Materials
Overnight culture of Serratia marcescens
20   grown in Luria Broth, static at 37°C.
Maximum Recovery Diluent (MRD) (Oxoid CM733,
Lot No. 203 40764) made up according to manufacturers
instructions.
Plates of Luria Agar (LA).
25   Plates of Violet Red Bile Glucose Agar
(VRBGA) (Oxoid CM485, Lot No. 138 40768) made up
according to manufacturers instructions.
Autoinducer stock: DL-N-(3-oxohexanoyl)-L-
homoserine lactone 10 mg/ml in ethyl-acetate.

30
Method
Plates with 20 ng Autoinducer/ml agar were
prepared in the following way:
Autoinducer solution 4 μg/ml was prepared by
35   mixing 4μl stock with 10 ml Maximum Recovery diluent.
Pre-poured, dried agar plates were weighted

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to nearest 2g, and 5 µl Autoinducer solution/g agar was
dispensed and spread on the surface using a sterile,
disposable L-shaped spreader. Plates were then stored
overnight at 4˚C in the dark before drying prior to

5   use.

Control plates without Autoinducer were
prepared in a similar fashion substituting sterile
Maximum Recover Diluent for Autoinducer solution.

The overnight culture was diluted in 10-fold

10 serial dilutions to 10^{-4} in sterile Bijou´s by mixing
500 µl culture + 4500 µl MRD (= 10^{-1}),
500 µl 10^{-1} + 4500 µl MRD (= 10^{-2})
500 µl 10^{-3} dilution + 4500 µl MRD (= 10^{-4}).

The 10^{-4} dilution was used in the further

15 work as working suspension.

Controls

100 µl working suspension was serially
diluted as described in 1000µl volumes, and 100 µl of

20 the resulting dilutions 10^{-1}, 10^{-2} and 10^{-3} were
surface plated in duplicate on each of the following
media:
Luria Agar (LA)
Luria Agar + 20 ng Autoinducer (LA + I)

25 Violet Red Bile Glucose Agar (VRBGA)
Violet Red Bile Glucose Agar + 20 ng Autoinducer
(VRGBA + I)

Freeze-shocked samples

30 The remaining 4900 µl of working suspension
was transferred to a freezer and stored at -21˚C.
After 210 minutes this was thawed in a waterbath at
31.5˚C for 15 minutes, and duplicates of 100 µl of the
thawed working solution and a 10^{-1} dilution in MRD of

35 the thawed working solution were plated on the 4 agars
as described.

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Plates were counted after incubation at 22˚C for 24 and 48 hrs.

Results

5   Results are given as colony forming units recovered per ml working solution. Table 4 shows culture densities on the different agars after 48 hours incubation.

Table 5 shows culture densities on Luria Agar
10   with and without Autoinducer after 24 and 48 hours incubation.

Conclusion

There is no difference between recovery of
15   un-injured cells on complex and selective media with and without autoinducer.

There is no difference between recovery of injured cells on complex medium (Luria Agar) with and without inducer after 48 hours, but there is a trend
20   towards quicker growth/recovery (higher number of colonies after 24 hours) on Luria Agar with Autoinducer compared to the same medium without Autoinducer.

There is a significant increase in recovery of injured cells on selective medium (VRGBA) with
25   inducer compared to VRGBA without inducer.

Recovery without inducer:
% recovery on VRGBA = 2.8/11 = 25%.

30.   Recovery with inducer:
% recovery on VRGBA + I = 5.1/11 = 46%.

Recovery on LA after 24 hrs:
With Inducer = 8.5/11 = 77%.
35   Without Inducer = 6.9/11 = 63%.

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Discussion

        The higher recovery of injured organisms on
the selective agar with Autoinducer indicates an
improvement in tolerance to selective agents (Bile
5   salts and Crystal Violet) and/or improved repair of
injured organisms.

        The higher number of colonies found after 24
hours on Luria Agar with Autoinducer compared to
controls without Autoinducer implies quicker
10   recovery/growth in the presence of Autoinducer.

Table 4

Culture densities on 4 different agars after 48 hours
15   at 22°C

Culture density 48 hrs incubation $(\times10^3)$	LA	VRGBA	LA + I	VRBGA + I
Control	27	28	29	30
Frozen	11	2.8	11	5.1

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Table 5

Culture densities on Luria Agar with and without
Autoinducer after 24 and 48 hours incubation at 22°C

| Culture density $(\times 10^3)$	LA 24 hrs	LA 48 hrs	LA + I 24 hrs	LA + I 48 hrs
Control	26	27	29	29
Frozen	6.9	11	8.5	11

- 42 -

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CLAIMS

1.   Use of a compound having the formula 1 to control gene expression in microorganisms other than _V. fischeri_, _V. logei_ and _V.harveyi_, wherein formula 1 is

$$R-NH-\overset{\displaystyle (CH_2)_n}{\underset{\underset{\displaystyle X}{\displaystyle \|}}{\diagup}}Y \qquad\qquad 1$$
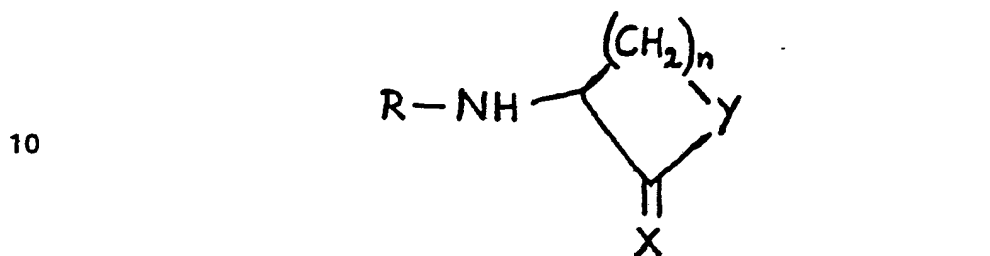
where n is 2 or 3

Y is O, S or NH

X is O, S or NH

R is $C_1 - C_{12}$ alkyl or acyl which may be substituted.

2.   Use as claimed in Claim 1, wherein, Y is O, X is O, n is 2 and R is acyl.

3.   Use as claimed in Claim 1 or Claim 2, wherein R carries a keto or hydroxy group.

4.   Use as claimed in Claim 3, wherein R carries a keto group in the beta-position.

5.   Use as claimed in Claim 4, wherein the compound is N-(β-ketocaproyl) homoserine lactone.

6.   Use as claimed in any one of Claims 1 to 5, wherein the gene expression causes bioluminescence.

7.   Use as claimed in any one of Claims 1 to 5, wherein the gene expression causes production of antibiotic.

8.   Use as claimed in any one of Claims 1 to 5, wherein the compound is included in a bacterial growth medium which promot s bacterial growth.

9.   An optically active isomer of a compound having formula 1 as defined in any one of Claims 1 to 5.

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10.   An optically active compound as claimed in
Claim 9, wherein the isomer is the L-isomer.

11.   A method of testing for N-(β-ketocaproyl)
homoserine lactone or analogue in a sample, by
5   incubating the sample in contact with test bacteria
chosen for enhancement of gene expression by the
lactone or analogue, and detecting the gene expression
as a test for the lactone.

12.   A method of testing for the presence in a sample
10   of a first bacterium known to generate N-(β-
ketocaproyl) homoserine lactone or analogue under
particular conditions, which method comprises
incubating the sample under the particular conditions
in contact with a test bacterium chosen for enhancement
of gene expression by the lactone or analogue, and
15   detecting the gene expression as a test for the first
bacterium.

13.   A method as claimed in Claim 12, wherein the
first bacterium is selected from Pseudomonas
20   aeruginosa, Serratia marcescens, Proteus mirabilis,
Citrobacter freundii and Enterobacter spp.

14.   A method as claimed in Claim 12 or Claim 13,
wherein a fluid sample possibly containing the first
bacterium is filtered, and a nutrient medium containing
25   the test bacterium is laid on the filter and incubated.

15.   A method as claimed in any one of Claims 11 to
14, wherein the gene expression causes bioluminescence.

16.   A method as claimed in any one of Claims 11 to
15, wherein gene expression causes production of
30   antibiotic.

17.   A growth medium for microorganisms,
containing an added compound having formula 1 as
defined in any one of claims 1 to 5, at a
concentration effective to stimulate or promote the
35   metabolism, growth and/or recovery of microorganisms.

FIG.1

REGULATION OF THE LUX OPERON OF Vibrio fischeri
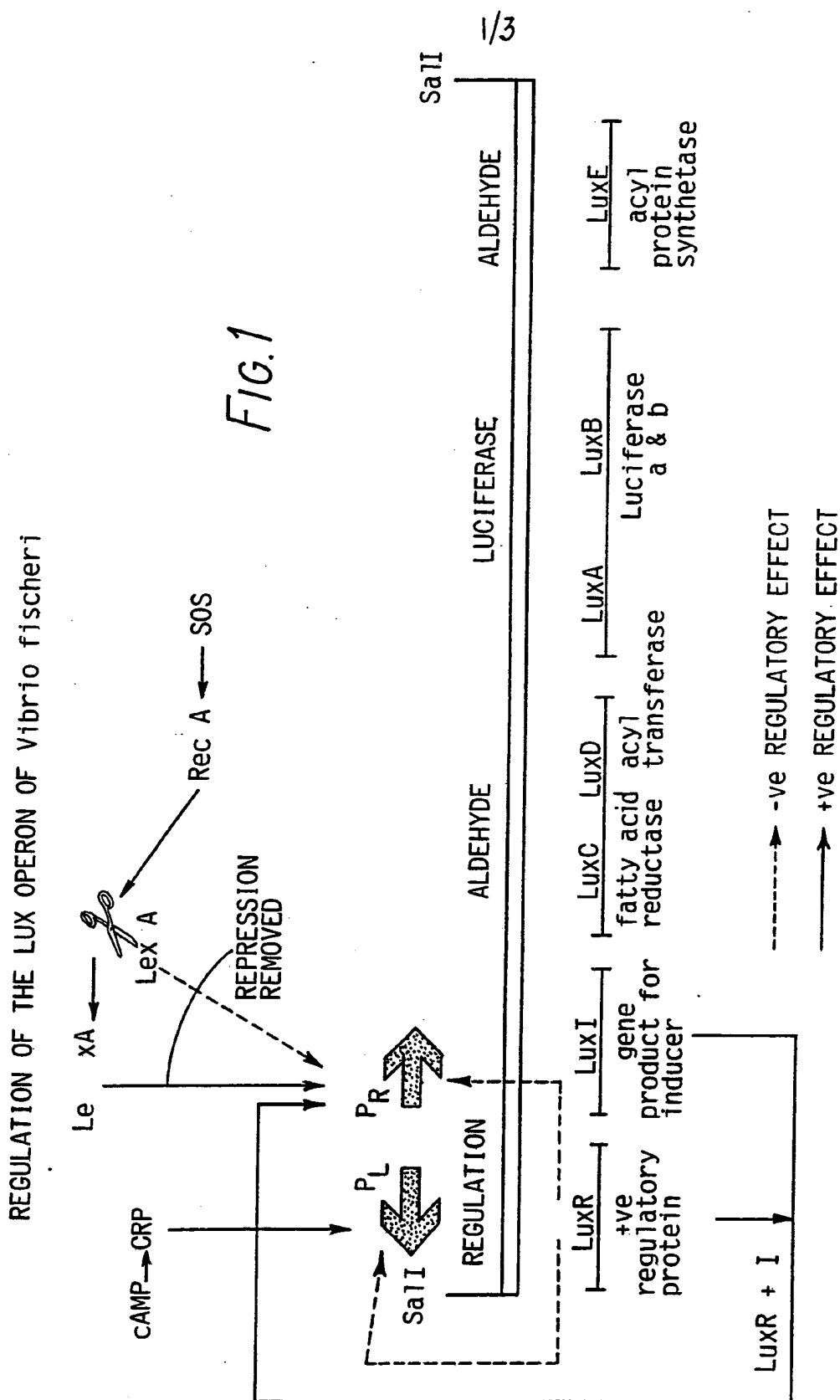
FIG.2   N-(B-ketocaproyl)-homoserine lactone
Vibrio fischeri autoinducer

INDUCER CONCENTRATION (ng)
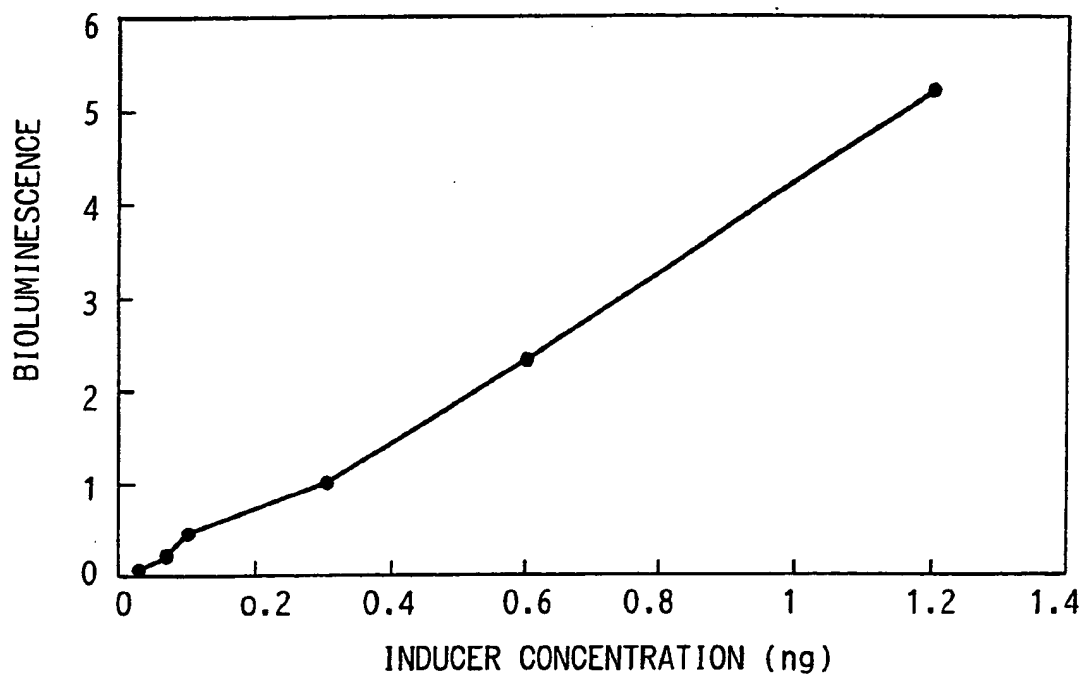
Light from E.coli(pSB237) after 24 h
incubation with autoinducer

FIG.3
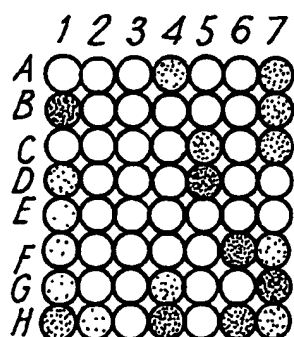
AFTER 6 HOURS OF
INCUBATION

FIG.5
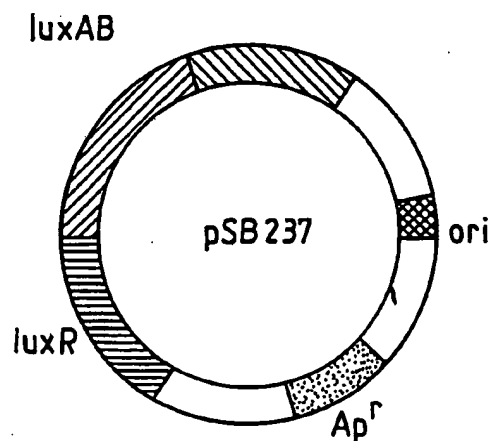
FIG. 4

DOSE-RESPONSE CURVES FOR SOME AUTOREGULATOR ANALOGUES
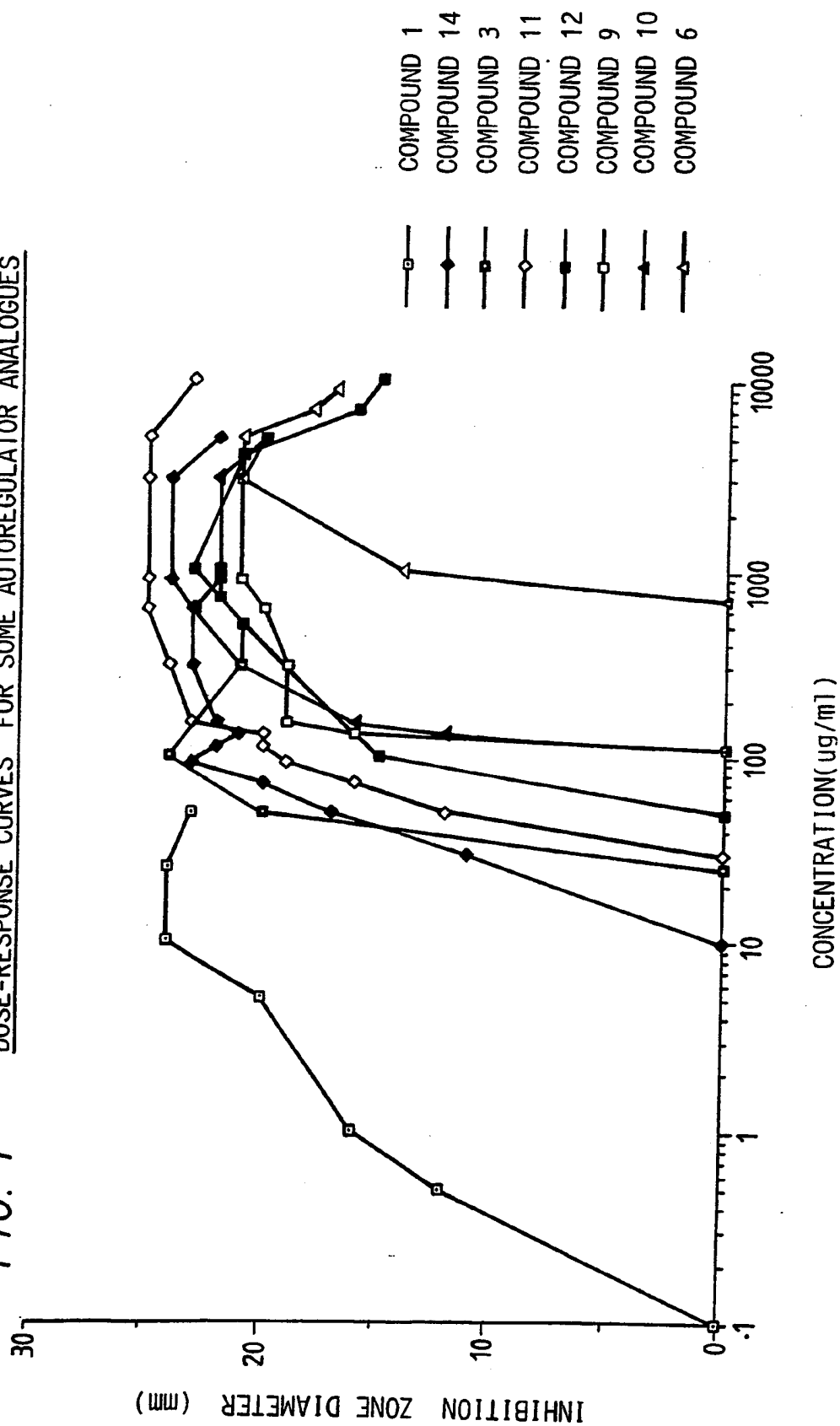
I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/38;   C12N15/67;   C12Q1/04;   C07D307/33
        C07D309/30;   C07D333/32;   C07D207/273;   C07D335/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12Q ; C07D

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	US,A,4 861 709 (ULITZUR, S.Y. & KUHN, J.C.) 29 August 1989 see column 7 - column 8; claim 10 ---	1-6,8,17
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, August 1989, WASHINGTON US pages 5688 - 5692; DEVINE, J.H. ET AL.: 'Identification of the operator of the lux regulon from the Vibrio fischeri strain ATCC7744' see the whole document --- -/--	1-6,8,17

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JULY 1992	16. 07. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4861709	29-08-89	None	

For more details about this annex : see   fficial Journal of the European Patent   ffice, No. 12/82

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